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ORIGINAL PAPER

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Pre-termination in *aflR* of *Aspergillus sojae* inhibits aflatoxin biosynthesis

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Abstract The *aflR* gene product is the main transcriptional regulator of aflatoxin biosynthesis in *Aspergillus parasiticus* and *Aspergillus flavus*. Although *A. sojae* strains do not produce aflatoxins, they do have an *aflR* homologue. When compared with the *aflR* of *A. parasiticus*, the *A. sojae* gene contains two mutations: an HAHA motif and a premature stop codon. To investigate the functionality of the *A. sojae aflR* gene product, we used a GAL4 one-hybrid system in yeast. The transcription-activating activity of AflR from *A. sojae* was 15% of that from *A. parasiticus*. The introduction of an additional *aflR* from *A. sojae* into an *A. parasiticus* strain did not affect aflatoxin productivity. A hybrid *aflR* comprising the amino-terminal region of *A. sojae aflR* and the carboxy-terminal region of *A. parasiticus aflR* suppressed the effect associated with pre-termination of the *A. sojae* AflR. We conclude that the premature stop codon of the *A. sojae aflR* is the key to its functionality and leads to prevention of aflatoxin biosynthesis through loss of the transcription of aflatoxin biosynthesis-related genes.

Introduction

Aflatoxin, one of the most potent naturally occurring carcinogens or mutagens, is produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Brown et al. 1999). These filamentous fungi belong taxonomically to *Aspergillus* section *Flavi* (Klich and Pitt 1988). This section also includes *Aspergillus oryzae* and *Aspergillus sojae*, which are used for the production of various industrial enzymes and fermented foods in eastern Asia. Although these fungi do not produce aflatoxins, they are taxonomically similar to aflatoxin-producing species (Kurtzman et al. 1986); therefore, it is necessary to determine whether these strains have the potential to make toxins. Because the genetics of aflatoxin synthesis have been studied extensively, we used molecular analyses to investigate the lack of aflatoxin production in *A. sojae*.

More than 20 genes are involved in aflatoxin biosynthesis, and these genes constitute a gene cluster in the genomic DNA of *A. parasiticus* and *A. flavus* (Yu et al. 1995). Among the clustered genes, *aflR* encodes a main transcriptional regulator of aflatoxin-related genes (Chang et al. 1993; Payne et al. 1993; Yu et al. 1996). The predicted AflR is a GAL4-type zinc-finger protein (Woloshuk et al. 1994; Chang et al. 1995) that binds to the 5′-flanking region of aflatoxin-related genes at specific AflR-binding elements (Chang et al. 1995; Ehrlich et al. 1999). This gene is essential for aflatoxin biosynthesis, since disruption of the *aflR* gene in aflatoxigenic *A. parasiticus* strains results in an inability to produce aflatoxin (Cary et al. 2000). The carboxy-terminal region of AflR is thought to be critical for transcriptional activation (Chang et al. 1999a).

Although *A. oryzae* and *A. sojae* strains do not produce aflatoxin, they contain homologues of *aflR* and other aflatoxin-related genes (Klich et al. 1995; Kusumoto et al. 1998; Matsushima 2001). Watson et al. (1999) reported two characteristic features in the *aflR* homologue of *A. sojae* (Fig. 1): (1) a duplication of the histidine and alanine residues at positions 111–114 (HAHA motif), and (2) a C→T transition that replaces Arg-385 with a stop codon, leading to truncation of the carboxy-terminal region by 62 residues.

To confirm the safety of *A. sojae* for soy sauce fermentation, the functionality of the *aflR* homologue from this industrial strain was examined. The transcription-

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a

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                    acg cct cat gct cat acc cag gcc cac act cat gct --- --- cat tct cat ccg caa ccg cat cca
A. parasiticus 101  T   P   H   A   H   T   Q   A   H   T   H   A   -   -   H   S   H   P   Q   P   H   P     120
strain 477     101  T   P   H   A   H   T   Q   A   H   T   H   A   H   A   H   S   H   P   Q   P   H   P     122
                    acg cct cat gct cat acc cag gcc cac act cat gct cat gct cat tct cat ccg caa ccg cat cca
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b

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                    cga gtg gcg gca cag ctt gtt ctg agt gaa ctg cac cga gtg cag tcg ctg gcg aac cta
A. parasiticus 371  R   V   A   A   Q   L   V   L   S   E   L   H   R   V   Q   S   L   A   N   L   390
strain 477     373  R   V   A   A   Q   L   V   L   S   E   L   H   *                               384
                    cga gtg gcg gca cag ctt gtt ctg agt gaa ctg cac tga gtg cag tcg ctg gcg aac cta
                                                                   =
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Fig. 1 a, b Comparison of the nucleotide and deduced amino acid sequences of the *aflR* genes of *Aspergillus parasiticus* and *Aspergillus sojae*. The GenBank accession number for *aflRap* is L26220. Like those of other *A. sojae* strains, the *aflR* gene of strain 477 had two characteristic features: the HAHA motif and pre-termination at Arg-385. The nucleotide and protein sequences surrounding these mutations are indicated. **a** The *aflR* gene of strain 477 contains a duplication of six nucleotides (*underlined*), forming a duplicated HA at His-113 and Ala-114. **b** The transition in the codon of Arg-385 (*double-underlined*) results in the pre-termination of AflR; the truncated protein lacks 62 amino acids, including the transcription-activating domain, at the carboxy-terminal end

activating activity of *A. sojae aflR* was determined by using a GAL4 one-hybrid system in yeast, and the effect of introducing *aflR* from *A. sojae* into *A. parasiticus* was evaluated.

Materials and methods

Fungal strains

Aspergillus sojae strain 477, a mutant of IFO4241 that showed high levels of protease production (Matsushima 2001), was used. The *A. parasiticus* strain was SRRC2043, which accumulates *O*-methylsterigmatocystin (OMST), an intermediate during aflatoxin biosynthesis (Chang 1995). To induce aflatoxin production, the cultures were grown on A and M medium (5% glucose, 0.3% $(NH_4)_2$ SO_4, 1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.7 mg/l $Na_2B_4O_7 \cdot 10H_2O$, 0.5 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 mg/l $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.3 mg/l $CuSO_4 \cdot 5H_2O$, 0.11 mg/l $MnSO_4 \cdot H_2O$, 17.6 mg/l $ZnSO_4 \cdot 7H_2O$) with shaking (150 rpm) at 29 °C for 3 days (Adye and Mateles 1964).

Cloning of *aflR* from strain 477

The *aflR* gene from *A. sojae* (*aflRas)* was cloned from the genomic library of strain 477, which was constructed by using Lambda EMBL3/Gigapack III Gold Cloning kit (Stratagene, La Jolla, Calif., USA). A PCR-derived 0.7-kb fragment of *aflRas* was used as the probe for cloning. The primers used to generate the probe were 5′-GAT AGC TGT ACG AGT TGT GC-3′ and 5′-CAG CCC AGC GGG GCG TGG GG-3′. The amplified DNA fragment was labeled with digoxigenin-11-dUTP by using the PCR DIG Probe Synthesis Kit (Boehringer-Mannheim, Mannheim, Germany). The *Sph*I–*Hin*dIII fragment including *aflRas* was subcloned into pUC18 to construct pARH7. The genomic clone of *aflR* did not contain any intron (Chang 1995).

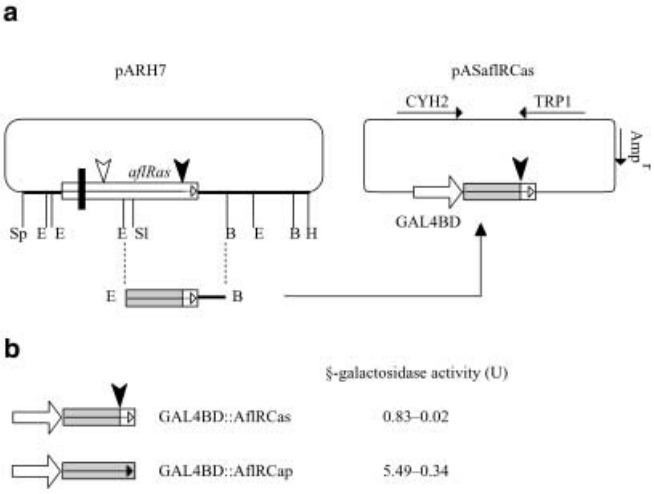

Fig. 2 a, b Transcription-activating activity of the carboxy-terminal region of AflR. **a** The construction of pASaflRCas is shown. The plasmid pASaflRCap was constructed by following the same procedure and using pHSp instead of pARH7. Sp, *Sph*I; E, *Eco*RI; Sl, *Sal*I; B, *Bam*HI; H, *Hind*III; *open arrowhead*, HAHA motif; *closed arrowhead*, premature stop codon; *closed box*, zinc finger motif. **b** Transcription-activating activities of various AflR carboxy-terminal regions are shown. The yeast transformant produces a fusion protein, GAL4BD::AflRCas or GAL4BD::AflRCap, consisting of a GAL4 DNA-binding domain (GAL4BD, *open arrow*) and the transcription-activating domain of AflR (*gray box*). The truncated region in the carboxy-terminal end is indicated as an *open box*. The β-galactosidase activities shown are the average±SD calculated from three transformants

Construction of *aflR* for one-hybrid expression

The plasmid pHSp contained the cloned *aflRap* (the *aflR* gene from *A. parasiticus*) fragment and *niaD* as a selectable marker (Chang et al. 1995). Plasmids pHSp and pARH7 were digested with *Eco*RI and *Bam*HI. Each 0.9-kb fragment was ligated to pAS2–1 (Clontech, Palo Alto, Calif., USA) to generate pASaflRCap and pASaflRCas (Fig. 2A).

The vector constructs were transformed into *Saccharomyces cerevisiae* Y190 by using the PEG-LiAc protocol (Chang et al. 1999a). Transformants were selected on Minimal Synthetic Drop Agar (Clontech) plates that were supplemented with all required amino acids except tryptophan. β-Galactosidase activity was determined by using a liquid culture assay in which *o*-nitrophenyl-β-D-galactopyranoside was the substrate (Chang et al. 1999a).

Introduction of additional *aflR* into *A. parasiticus* and *A. sojae*

The *Sph*I–*Hin*dIII fragment containing the *aflRap* portion of pHSp was replaced with the *aflRas*-containing *Sph*I–*Hin*dIII fragment of

Table 1 Functionality of *Aspergillus sojae* AflR in the production of aflatoxin precursors. *AVN* Averantin, *OMST O*-methylsterigmatocystin

Host[a]	Endogenous *aflR*	Introduced *aflR*[b]	Aflatoxin intermediates (μg/mg of mycelial dry weight)[c]	
			AVN	OMST
A. sojae	*aflRas*	None	ND[d]	ND
A. parasiticus	*aflRap*	None	2.26	23.24
A. sojae	*aflRas*	*AflRas*	ND	ND
A. parasiticus	*aflRap*	*AflRas*	3.13±0.60	26.95±0.18
A. parasiticus	*aflRap*	Hybrid[e]	28.36±4.30	72.66±2.35

[a] The *niaD* mutant of strain 477 was the *A. sojae* strain used, and the *niaD* mutant of SRRC2043 was the *A. parasiticus* strain used
[b] Exogenous genes were integrated at the *niaD* locus
[c] Data reported represent average±SD calculated from three transformants
[d] Not detected
[e] The carboxy-terminal region of the *A. sojae aflR* was replaced with the corresponding region of *aflRap*

pARH7. The resulting plasmid was used to transform *niaD* mutants of *A. parasiticus* SRRC2043 and *A. sojae* strain 477 as described previously (Horng et al. 1990).

The carboxy-terminal region of *aflRas* was excised from pARH7 by using *Sal*I and *Hin*dIII and replaced with the corresponding *Sal*I–*Hin*dIII fragment from pHSp. The generated plasmid was introduced into the *niaD* mutant of *A. parasiticus* SRRC2043 as described. Integration of vectors was confirmed by Southern analysis (data not shown).

Determination of metabolites

Averantin (AVN) and OMST, intermediates of aflatoxin biosynthesis that were produced by the fungal cultures, were extracted with chloroform, separated by thin-layer chromatography, and quantified as described previously (Cleveland 1991).

Results

Cloning and sequence analysis of *aflR* from *A. sojae*

Sequencing of pARH7 revealed that the nucleotide sequence of *aflR* from strain 477 was identical to that of *aflRas* (GenBank accession number Y16967; Watson 1999). Furthermore, the two features characteristic of the deduced protein from *aflRas*, AflRas, the HAHA motif and the truncated carboxy-terminal region, were conserved (Fig. 1).

Transcription-activating activity of AflRas

To test whether AflRas had transcription-activating activity, the *GAL1*::*lacZ* gene expression system in *Saccharomyces cerevisiae*, in which transcription was activated by the carboxy-terminal region of the deduced protein from *AflRap*, AflRap, was used. Transformants resulting from the introduction of pASaflRCap and pASaflRCas produce the fusion proteins GAL4BD::AflRCap and GAL4BD::AflRCas, respectively, which carry a GAL4 DNA-binding domain and the transcription-activating domain of AflRap or AflRas (Fig. 2B). The transcriptional activity was evaluated by the β-galactosidase activity (Chang et al. 1999a). The β-galactosidase activity of transformants harboring pASaflRCas was 15% of that observed in pASaflRCap-containing transformants (Fig. 2B). Thus, truncation of the carboxy-terminal region of AflRas reduced the transcription-activating activity for *GAL1*::*lacZ* expression in yeast, although it did not disappear.

Influence of additional *aflR* on aflatoxin production in *A. parasiticus* and *A. sojae*

To investigate its functionality in vivo, we introduced an *aflRas* gene into *niaD* mutants of *A. parasiticus* SRRC2043 and *A.sojae* strain 477; these strains contain an endogenous *aflR* gene (Table 1). Introduction of *aflRas* into SRRC2043 did not increase the production of intermediates of aflatoxin biosynthesis. Similarly, introduction of additional *aflRas* into *A. sojae* strain 477 failed to stimulate production of aflatoxin-associated intermediates.

We replaced the carboxy-terminal region of *aflRas* with its counterpart from *aflRap*. The resultant *aflR* yielded the hybrid AflR, with the amino-terminal portion from *A. sojae* and the carboxy-terminal portion from *A. parasiticus*. This hybrid construct was introduced into the *niaD* mutant of *A. parasiticus* SRRC2043 (Table 1). Expression of the exogenous hybrid *aflR* increased the production of aflatoxin precursors in the transformants to levels similar to in transformants carrying the exogenous wild-type *aflRap* (Table 1; Chang et al. 1995).

Discussion

Aspergillus sojae strains are used for industrial food fermentation, such as soy sauce and bean paste production. Although they do not produce aflatoxins, *A. sojae* strains are taxonomically similar to aflatoxigenic fungi and maintain genes associated with aflatoxin biosynthesis (Matsushima et al. 2001). Therefore, further molecular characterization of these fungi is necessary to confirm their safety. We have been investigating the absence of aflatoxin production in strain 477(Matsushima et al.

2001), which is currently used for the fermentation of soy sauce.

Sequence analysis revealed that, compared with *aflRap*, *aflRas* contains two distinct mutations: the HAHA motif and a premature stop codon (Fig. 1). These mutations are conserved characteristics among the *A. sojae* strains evaluated previously (Watson 1999). The product of *aflR* is a transcriptional regulator of aflatoxin biosynthesis in *A. parasiticus* and *A. flavus* (Woloshuk et al. 1994; Chang et al. 1995). With a few exceptions (Klich et al. 1997), strains classified to *A. sojae*, including strain 477, do not transcribe *aflR* (Kusumoto 1998; Matsushimae et al. 2001). We investigated the functionality of *aflRas* to determine whether these mutations contribute to the lack of aflatoxin biosynthesis in *A. sojae*.

The premature stop codon in *aflRas* would result in a truncated protein that lacks 62 residues, including the transcription-activating domain. The assay using the GAL4 one-hybrid system showed that the transcription-activating activity of the carboxy-terminal region of AflRas was 0.83 U, which was only 15% of that of AflRap (Fig. 2B). The last 22 amino acids in the carboxy-terminal region form the putative transcription-activating domain of AflRap. Deletion of this region reduced the transcription-activating activity of the resulting protein to less than 0.7 U (Chang 1999a), a finding that is consistent with our results. The truncation in AflRas may hamper its effectiveness as a transcriptional activator.

Introducing an additional copy of *aflRap* into *A. parasiticus* increases the production of aflatoxin and its precursors (Chang 1995). We examined this gene-dosage effect of *aflRas* for aflatoxin production. Introducing an additional copy of *aflRas* into *A. sojae* failed to stimulate aflatoxin biosynthesis. Furthermore, the introduction of *aflRas* into *A. parasiticus* SRRC2043 did not increase the production of aflatoxin intermediates (Table 1). Thus, it appears that *aflRas* is incapable of activating aflatoxin biosynthesis in either *A. parasiticus* or *A. sojae.*

Pre-termination of translation reduced the transcription-activating activity of AflRas (Fig. 2). To prove that the loss of activity was due to the truncation at the carboxy-terminal region, we constructed a hybrid *aflR* that carried the mutant HAHA motif but a normal carboxy-terminal region. In transformants carrying the hybrid *aflR*, production of aflatoxin precursors increased to levels similar to those induced by wild-type *aflRap* (Chang et al. 1993, 1995). The HAHA motif in the amino-terminal region is not important for AflR function because, except for its mutant HAHA motif, the hybrid AflR was identical to AflRap.

The pre-termination of AflRas may be the definitive mutation affecting the functionality of AflR and aflatoxin biosynthesis. Although AflRas retained some transcription-activating activity in the yeast system we used, the level of activity might be insufficient to promote transcription of aflatoxin-related genes (including *aflR* itself) in fungal cells. The upstream region of *aflR* contains AflR-binding elements, and the *aflR* gene is auto-regulated (Chang et al. 1995; Ehrlich et al. 1999). From these observations we speculated that the poorly active AflRas, with its truncated carboxy-terminal region, decreases transcription and expression of *aflRas* to a level lower than the threshold required for activating transcription of aflatoxin-related genes. Furthermore, the carboxy-terminal region of AflRap interacts with putative repressors of *aflR* and breaks the repression, thereby enhancing the transcription of *aflR* (Chang 1999b). The truncated AflRas may fail to interact with these repressors, so that the *aflRas* gene remains repressed and untranscribed in *A. sojae*.

We showed that AflRas had lost its functionality for aflatoxin biosynthesis because of the premature stop codon in *aflRas*. However, the absence of transcription of *aflRas* (Matsushima et al. 2001) implies other defects in *A. sojae*. Thus, while a nonfunctional AflRas may guarantee the safety of *A. sojae* strains in terms of their industrial use, the mechanism behind the inability of *A. sojae* strains to synthesize aflatoxins is still unclear. Further studies to answer this question are in progress.

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